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Caveolin-1 Regulation of *Disrupted-in-Schizophrenia*-1 as a Potential Therapeutic Target for Schizophrenia

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Abstract

Background: Schizophrenia is a debilitating psychiatric disorder manifested in early adulthood. *Disrupted-In-Schizophrenia-1* (*DISC1*) is a susceptible gene for schizophrenia (1-3) implicated in neuronal development, brain maturation and neuroplasticity (4, 5). Therefore, DISC1 is a promising candidate gene for schizophrenia, but the molecular mechanisms underlying its role in the pathogenesis of the disease are still poorly understood. Interestingly, Caveolin-1 (Cav-1), a cholesterol binding and scaffolding protein, regulates neuronal signal transduction and promotes neuroplasticity. Here we examined the role of Cav-1 in mediating DISC1 expression in neurons *in vitro* and the hippocampus *in vivo*.

Methods and Results: Overexpressing Cav-1 specifically in neurons using a neuron specific synapsin promoter (*SynCav1*) increased expression of DISC1 and proteins involved in synaptic plasticity (PSD95, synaptobrevin, synaptophysin, neurexin, and syntaxin-1). Similarly, SynCav1-transfected differentiated human neurons derived from induced pluripotent stem cells (hiPSCs) exhibited increased expression of DISC1 and markers of synaptic plasticity. Conversely, hippocampi from Cav-1 knockout (KO) exhibited decreased expression of DISC1 and proteins involved in synaptic plasticity. Finally, *SynCav1* delivery to the hippocampus of Cav-1 KO mice and Cav-1 KO neurons in culture restored expression of DISC1 and markers of synaptic plasticity. Furthermore, we found that Cav-1 co-immunoprecipitated with DISC1 in brain tissues.

Conclusion: These findings suggest an important role by which neuronal Cav-1 regulates DISC1 neurobiology with implications for synaptic plasticity. Therefore, *SynCav1* might be a potential therapeutic target for restoring neuronal function in schizophrenia.

Introduction

Schizophrenia is one of the least understood debilitating psychiatric illnesses. Typically manifested in late adolescence or early adulthood, schizophrenia has an estimated prevalence of ~1% (6). Schizophrenia is partly a genetic disorder, although it likely involves multiple recessive genes, and environmental factors such as physical or psychological abuse and birth complications (7-9). While pharmacological treatments such as antipsychotics are available for schizophrenia, these classes of drugs show poor efficacy for most patients (10), especially in reversing cognitive abnormalities (11, 12).

DISC1 is a schizophrenia associated gene originally identified in a Scottish family (1, 3, 13), and later studies have shown an increasing amount of evidence that supports the possibility that DISC1 may be one of the candidate genes for schizophrenia (2, 14-20). DISC1 protein is highly expressed in the developing brain (21) and in the dentate gyrus of the adult hippocampus (22); it is a multifunctional protein involved in neuritogenesis and neuronal signaling (23-25). DISC1 is located in multiple intracellular locations (i.e., the nucleus (26), mitochondria (27), and in axons and synapses (28, 29)). Loss of DISC1 function causes deficits in neural development, neuronal proliferation, axonal growth, and cytoskeleton modulation, which are consistent with abnormal neural development in schizophrenia (30-32).

Proper neuronal growth (i.e., dendritic arborization, axonal guidance, and formation of synaptic contacts) and neurotransmission are dependent upon a polarized membrane platform that organizes key membrane receptors, which in turn transduce extracellular cues. A necessary organizer of neuronal signaling components is the scaffolding protein caveolin-1 (Cav-1) (33, 34). Cav-1 is widely expressed in the central and peripheral nervous systems (35, 36). Within neurons, Cav-1 regulates membrane/lipid raft formation and neurotransmitter and neurotrophin signaling (34), promotes dendritic growth and arborization (33), and when over-expressed in hippocampal neurons *in vivo*, augments functional neuroplasticity and improves learning and memory (37). To date no relevant functional role of Cav-1 in the pathogenesis of schizophrenia has been described, *CAV1* gene disruption was recently identified in some patients suffering from schizophrenia (38). Because Cav-1 organizes and regulates neurotransmitter and neurotrophic receptor signaling pathways (39-41) and G protein-coupled neurotransmitter receptors (42-44) necessary for proper dendritic growth and arborization (33, 37), disruption of *CAV1* would likely impair neuronal signaling leading to a schizophrenia-like phenotype.

Interestingly, recent findings supported the involvement of caveolin in schizophrenia; for instance, CNS pathologies in Cav-1 knockout (Cav-1 KO) mice are similar to those found in schizophrenia (34, 45) as Cav-1 KO mice exhibit increased sensitivity to the psychotomimetic effects of *N*-methyl-aspartate receptor antagonist phencyclidine (PCP) (46), a phenomenon also observed in schizophrenic patients (47, 48). Additionally, Cav-1 interacts with 5-HT_{2A} (44), a target for atypical antipsychotic drugs (49). Interestingly, Cav-1 KO mice showed an attenuated biochemical and behavioral actions of atypical antipsychotic drugs (46). These findings provide support linking Cav-1 to schizophrenia.

In the present study, our goal was to examine if Cav-1 impacts expression of DISC1. Interestingly, treatment of primary neurons with *synCAV1* lentivirus significantly enhanced the expression of DISC1. Furthermore, hiPSCs neurons overexpressing Cav-1 show higher DISC1 levels. Interestingly, hippocampal homogenates from Cav-1 KO mice showed a significant reduction in DISC1. Additionally, synaptic proteins such as PSD95, synaptophysin, synaptobrevin and syntaxin 1 were significantly reduced in the homogenates obtained from the hippocampi of Cav-1 KO mice compared to WT, where rescue of hippocampal Cav-1 resulted in rescue of synaptic protein expression.

Materials and methods

Animals

All animals (C57BL/6 mice and rats from Jackson Laboratories, Bar Harbor, Maine) were treated in compliance with the Guide for the Care and Use of Laboratory Animals (National Academy of Science, Washington, DC). All animal use protocols were approved by the Veterans Administration San Diego Healthcare System Institutional Animal Care and Use Committee

(San Diego, California) before any procedures were performed. Adult male mice (2-3 months old) were housed under normal conditions with *ad libitum* access to food and water.

Stereotactic Injection

Mice were anesthetized and prepared for surgery with a protocol modified from a previously described study (37). Hippocampal-targeted injections were controlled using Injectomate software (Neurostar, Berlin, Germany). Injections were made using a 33-gauge, 10-µL Hamilton gas tight syringe (Hamilton, Reno, Nevada). At each coordinate, the needle was lowered at a rate of 0.32 mm per second. After 60 seconds, 0.5 µL of adeno-associated virus serotype 9 containing synapsin-red fluorescent protein (RFP) (*SynRFP*) or synapsin-caveolin-1 (*SynCav1*) was injected over 60 seconds (0.5 µL/min injection rate at a viral titer of 10^9 genome copies (gc)/µL) at three locations (rostral to caudal) in each hippocampal hemisphere with an indwelling time of 1 minute. Sagittal brain sections were stained to confirm location and spread of RFP (data not shown). Sections were also stained for hematoxylin and eosin, and histopathologic analysis did not reveal any gross morphology or cell death in the hippocampal sections (data not shown).

Primary neuron isolation and culture

Neonatal rat neurons were isolated from hippocampi using a papain dissociation kit (Worthington Biochemical, Lakewood, NJ) as previously described (33, 34). Neurons were cultured in neuobasal A media supplemented with B27 (2%), 250 mM GLUTMax1, and penicillin/streptomycin (1%). Cells were cultured on poly-D-lysine/laminin (2 µg/cm^2) coated plates at 37°C in 5% CO_2 for 4 d prior to transfection with lentiviral vectors containing the synapsin promoter up-stream of the *CAV1* gene (*SynCav1*). *SynGFP* was used as control vector.

Titer for both vectors was 10^9 infectious units (i.u.) per ml.

The human neurons were differentiated from the CV4a neuronal stem cells (NSC). Cells were first grown on poly-L-Ornithine/Laminin coated plates. Cells were maintained in NPC base media (DMEM/F12, N2, B27, and penicillin/streptomycin) supplemented with b-FGF (20 ng/ml). Cells were then differentiated for 3-4 weeks in differentiating media (NPC base media supplemented with BDNF, GDNF and Dibutyryl-cAMP). Neurons were then infected with *SynCav1* or *SynGFP* as control.

Immunoblot Analysis

Cell lysates were prepared in buffer (50 mM Tris-HCl; 150 mM NaCl; pH 7.4) supplemented with protease and phosphatase inhibitors cocktail (Cell Signaling, Beverly, MA, USA). After 30 min incubation on ice, the cells were homogenized by a 23-gauge needle and the lysates were cleared of debris and unbroken cells by centrifugation (800 g, 5 min at 4°C). Protein concentrations were determined by the Bio-Rad protein assay (BIO-RAD Laboratories, Hercules, CA, USA). Equal amounts of cell lysates (10 μg) were loaded to determine expression of Cav-1, PSD95, neurexin (BD Biosciences, Franklin Lakes, NJ, USA), syntaxin1, synaptobrevin, synaptophysin (Abcam, Cambridge, MA, USA), and DISC1 (Thermo Fisher Scientific, Waltham, MA, USA). All protein expression was normalized to GAPDH (Cell Signaling Technology, Danvers, MA, USA). Horseradish peroxidase (HRP) conjugated secondary antibodies were from Santa Cruz Biotechnology. Immunoblots were subsequently detected by lumigen ECL Ultra (Lumigen, Southfield, Michigan, USA).

Immunoprecipitation

Immunoprecipitation against Cav-1 and DISC1 was performed using protein A agarose (Roche, Life Science) according to the manufacturer protocol. In brief, brain samples were homogenized in lysis buffer. The supernatants were collected by centrifugation at 10.000 g for 10 min at 4°C. Lysates were then incubated with antibodies at 4°C for 3 h, followed by and overnight incubation of Agarose A. The immunoprecipitates were analysed for the presence of cav-1 and DISC1 by western blot.

Statistical analysis

Results are expressed as mean ± SEM and analyzed using the GraphPad Prism 6 software (GraphPad Software, Inc., San Diego, CA, USA). T-tests, one way ANOVA and 2-way ANOVA were used to compare certain paired parameters. Values of $p < 0.05$ were considered significant.

Results

Cav-1 interacts and co-immunoprecipitates with DISC1

Given no reported links between Cav-1 and DISC1, we first sought to determine if Cav-1 interacts with DISC1. Our data indicate that Cav-1 co-immunoprecipitated with DISC1 (**Figure 1A,B**).

Neuron-targeted overexpression of Cav-1 enhances expression of DISC1 and synaptic proteins in primary neurons

To explore the implication of Cav-1 and DISC1 on the synaptic integrity, we overexpressed Cav-1, by transfecting primary neurons isolated form hippocampi from neonatal rats with *SynCav1* lentivirus, and we studied the expression of DISC1. Our immunoblot analysis showed that Cav-1

was successfully over-expressed **(Figure 2A,B)**. Additionally, our results showed a significant increase in the level of DISC1 with over-expression of Cav-1 **(Figure 2A,C)**. Furthermore, we wanted to examine the role of Cav-1 in the regulation of synaptic density proteins in primary neurons. Western blot data indicate that overexpressing Cav-1 in primary neurons resulted in increased expression of synaptic proteins (PSD95, synaptobrevin, syntaxin-1, and neurexin) **(Figure 2A, D-G)**.

Hippocampi from Cav-1 KO mice exhibit decreased expression of DISC1 and synaptic proteins

Because data in Figure 1 showed that Cav-1 overexpression increased expression of DISC1 and synaptic proteins, we tested whether the opposite occurred in brain tissue deficient in Cav-1. Indeed, immunoblot data showed that loss of Cav-1 was associated with decreased protein expression of DISC1 **(Figure 3A,B)** and the synaptic proteins neurexin-1, synaptobrevin, PSD95, synaptophysin and syntaxin **(Figure 3 A, C-G)**. Interestingly, loss of Cav-1 seemed to affect both post and pre-synaptic proteins suggesting decreased synaptic strength(50-52).

Re-expressing Cav-1 in Cav-1 KO mice increases DISC1 and synaptic proteins

To elucidate whether re-introducing Cav-1 could reverse the effect seen with loss of Cav-1, Cav-1 KO mice underwent stereotactic injections of *AAV9-SynCav1*. Successful over-expression of Cav-1 in the hippocampus was confirmed by immunoblot **(Figure 4A,B)**. Interestingly, re-expression of Cav-1 in Cav-1 KO hippocampi significantly increased the levels of DISC1 expression **(Figure 4A,C)** as well expression of pre- and post-synaptic proteins like PSD95, synaptophysin, synaptobrevin and neurexin-1 **(Figure 4A, D-H)**.

Neuron-targeted overexpression of Cav-1 enhances expression of DISC1 and synaptic proteins in differentiated human neurons derived from induced pluripotent stem cells (iPSCs)

To investigate whether the effect of Cav-1 on DISC1 and synaptic protein could be extrapolated to hiPSCs, primary human fibroblasts were reprogrammed into hiPSCs and subsequently differentiated into neurons. Interestingly, our data indicated that overexpressing Cav-1 in iPSC neurons **(Figure 5A,B)** was associated with an increased level of DISC1 **(Figure 5A,C)**. Additionally, immunoblot data showed a significant increase in synaptic protein markers (synaptobrevin, PSD95, synaptophysin, syntaxin 1A, and neurexin) after Cav-1 over-expression **(Figure 5A, D-H)**.

Discussion

DISC1 is a promising candidate for mental illnesses and synaptic regulation (5). Cav-1 is also essential for maintaining and stabilizing proper synaptic signaling (34). Over the past decade, there has been progress in understanding the neurobiology of schizophrenia, mainly by the identification of different susceptible gene factors (20, 53, 54). The present study is the first to definitively demonstrate that genetic manipulation of the scaffolding protein Cav-1 directly regulates expression of DISC1, a schizophrenia associated gene.

We have previously shown that increase in Cav-1 expression enhances signaling and promotes neuronal survival and growth (33). Interestingly, loss of Cav-1 accelerates neurodegenration (52) and Cav-1 KO mice have behavioral deficits and many schizophrenia-like symptoms such as altered motor function, altered emotion as well as memory deficits (34, 45, 55,

56). Furthermore, hippocampi from Cav-1 KO mice have reduced cerebrovascular volume and large reduction in neurons (52). It is also reported that Cav-1 KO mice are resistant to atypical antipsychotic drugs (46). In this context, Cav-1 is a scaffold for D_2-dopamine and 5-HT_{2A} receptors, which represent canonical targets for typical and atypical antipsychotic drugs (44, 49, 57, 58). These findings suggest the possibility that restoring Cav-1 could be one way to reduce non-responsiveness to antipsychotics. Cav-1 KO mice also exhibited increased sensitivity to psychomimetic effects of phencyclidine (PCP), a phenomenon observed in patients with schizophrenia (47, 48). For instance PCP significantly disrupted PPI in Cav-1 KO mice, and increased locomotor activity (46).

DISC1, an intracellular scaffold protein with many proteins interactions (8, 59-63), is essential for neuronal growth, regulation of early brain development, and synaptic formation and maintenance (23-25). DISC1 has been associated with a number of mental illnesses (64). DISC1 is highly expressed in the hippocampus (22) and downregulation of DISC1 in the adult dentate gyrus leads to abnormal morphological development and mispositioning of new dentate granule cells (65). Interestingly, impairment of adult hippocampal neurogenesis has been reported in schizophrenia (66). Furthermore, DISC1 mouse models display synaptic pathologies (67) and show cognitive deficits reflecting the ones found in schizophrenia such as impaired working memory (68, 69). The importance of DISC1 in synaptic function comes from its interaction with many proteins enriched in the synapses that regulate synaptic maturation and plasticity (59, 60). Knockdown of DISC1 in new-born granule cells in adulthood also leads to defects in axonal targeting and development of synaptic outputs (70). Indeed, DISC1 was been found to localize in the synapse in human postmortem samples (28), as well as mice and rats (71-73). More

specifically, DISC1 has been shown to be enriched in the postsynaptic density (PSD) fraction (71, 72, 74).

Synaptic plasticity is a dynamic mechanism that regulates synaptic function and, therefore, the information flow between presynaptic and postsynaptic neurons. Neural circuit functions rely mainly on the synaptic plasticity, and alteration in synaptic plasticity is responsible for many neurological and neuropsychiatric diseases (75-80). In fact, the cognitive deficits and positive symptoms manifested in schizophrenia suggest that there is impairment in the information processing performed by neural circuits within the brain (81, 82). The cognitive deficits in schizophrenia range from impaired sensory processing to deficits in the cognitive control mechanisms necessary to manage and organize information (83-85). Interestingly, risk genes and genetic mutations identified in schizophrenia patients are involved in synaptic function (86-90). Synaptic proteins, such as PSD95, synaptobrevin, syntaxin-1A and neurexin, are important for formation and proper function of post-synaptic densities, thus maintaining high fidelity neurotransmission (91-93). Similar studies on putative mouse models of schizophrenia have strongly suggested synaptic dysfunction (86, 89, 94-96). Abnormalities in the functional connectivity between micro-circuits in different brain regions are considered to be an important pathophysiological mechanism underlying dysfunction in schizophrenia and functional imaging and EEG studies in schizophrenia as well as mouse models support this possibility (75, 76, 78, 97-102). Furthermore, *in vivo* plasticity studies conducted in persons with schizophrenia have shown reduction or complete absence of long-term plasticity evoked by transcranial stimulation (103-106). By understanding the basic pathophysiological mechanisms of cognitive decline and how the subcellular organization of key synaptic molecules is altered, we hope to better

understand the cellular and molecular mechanisms that may underlie schizophrenia and other neuropsychiatric diseases.

Although schizophrenia is a complex genetic and environmental disorder, not characterized by a single cause, it is the functional effect that will lead to the development of the illness by impairing synaptic plasticity and neuron interactions. We believe that by maintaining the proper function of neurons, and restoring the levels of synaptic proteins, there may be potential to conceivably treat the disease. Pharmacological treatments for schizophrenia are mainly antipsychotic drugs exerting their effects through blockade of the type 2 dopaminergic receptor (107). The use of these drugs for treatment is based on 60 year-old mechanism and have severe side effects. The non-efficacy of these drugs suggests a need to discover novel therapeutic targets and approaches to deliver these therapies.

A limitation of the present study is a lack of understanding of the cellular mechanism through which Cav-1 regulates DISC1 expression. Previous work from our group has shown that Cav-1 co-localizes with NMDARs, and loss of Cav-1 disrupts NMDAR-mediated signaling, NMDAR-mediated cAMP production, and NMDAR-mediated neuroprotection against oxygen-glucose deprivation (33, 34). Interestingly others have shown in certain mouse models involving reduced NMDAR expression, that these mice also have decreased DISC1 levels and exhibit schizophrenia-like mental disorders such as increased motor activity and deficits in social and sexual interactions (108). The modulation of DISC1 by Cav-1 could also involve cAMP signaling. Previous studies have shown that DISC1 regulates cAMP production through its interaction with certain phosphodiesterases (PDE4) in post-synaptic densities (109-111). Based upon our past and current findings that *SynCav1* increases NMDAR and DISC1 expression, and augments NMDAR-mediated cAMP production (33), it is conceivable that *SynCav1* could

potentially reverse the schizophrenia-like behavioral phenotype in a DISC1/cAMP-dependent signaling pathway. More experiments need to be conducted to confirm this hypothesis.

In summary, the present findings demonstrate an important role for Cav-1 in regulating DISC1 expression and maintaining synaptic proteins essential for neuroregeneration, synapse formation and function. Neuronal Cav-1 maybe a control point in neurotransmission and neuromodulation that is impaired in schizophrenia. Further understanding of how Cav-1 and DISC1 interact to maintain and organize neuronal growth, signaling and proper function is of upmost importance to better understand and identify potential molecular targets for treating schizophrenia.

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Legends

Figure 1. Caveolin-1 (Cav-1) is an interaction partner of Disrupted-In-Schizophrenia 1 (DISC1)

Brain homogenates were immunoprecipitated with a Cav-1 or DISC1 antibodies. Immunoprecipitates were then probed for the presence of Cav-1 and DISC1 by western blotting. Negative controls are incubated without antibodies.

Figure 2. Neuron-targeted expression of Caveolin-1 (Cav-1) enhances expression of Disrupted-In-Schizophrenia-1 (DISC1) and synaptic proteins in wild-type (WT) primary neurons.

Primary rodent neurons were isolated from rats' neonatal hippocampi. Neurons were grown in culture for 4 days and infected with a lentivirus containing the SynCav-1 or SynGFP as control ($2x10^9$ viral particles) for 72h. Homogenates were immunoblotted for Cav-1, DISC1, PSD95, Synaptobrevin, Syntaxin1, Neurexin, Synaptophysin and GAPDH **(A)**. Western blots quantification showed over-expression of Cav-1 protein **(B)**. Syncav1 also significantly enhanced the protein expression of DISC1 **(C)**, and other synaptic proteins: PSD95 **(D)**, Synaptobrevin **(E)**, Syntaxin1 **(F)**, and Neurexin **(G)**. ***p<0.05 between SynCAV1 and SynGFP.**

Figure 3. Hippocampal homogenates show a caveolin dependent reduction in Disrupted-In-Schizophrenia-1 (DISC1) and synaptic proteins.

Hippocampi were isolated from the brains of Wild-type (WT) and caveolin-1 knock-out (Cav-1 KO) mice (2-3 months). Homogenates were immunoblotted for Cav-1, DISC1, PSD95, Synaptobrevin, Syntaxin1, Neurexin, Synaptophysin and GAPDH **(A)**. Western blots quantification showed a significant decrease in the protein expression level of DISC1 **(B)**, and other synaptic proteins: Neurexin **(C)**, Synaptobrevin **(D)**, PSD95 **(E)**, Synaptophysin **(F),** Syntaxin1 **(G)**. ***p<0.05 between WT and Cav-1 KO.**

Figure 4. Neuron-targeted expression of caveolin-1 (Cav-1) enhances expression of Disrupted-In-Schizophrenia-1 (DISC1) and synaptic proteins in caveolin-1 knockout (Cav-1 KO) mice hippocampi.

2 months old Cav-1 KO mice were subjected to stereotactic injection of Associated Adeno virus9 (AAV9) containing SynCav-1 or SynRFP (as control), wild-type (WT) mice served as basal control. Mice were sacrificed one moth later and hippocampi were collected and homogenized. Homogenates were immunoblotted for Cav-1, DISC1, PSD95, Synaptobrevin, Syntaxin1, Neurexin, Synaptophysin and GAPDH **(A)**. Western blot quantification showed that SynCav-1 injection restored the Cav-1 expression **(B)** and significantly increased the expression levels of DISC1 **(C)**, and other synaptic proteins: PSD95 **(D)**, Synaptophysin **(E)**, synaptobrevin **(F)** and neurexin **(G)**. ***p<0.05 between WT vs SynCAV1 and WT vs SynRFP.** $^{\#}$**p<0.05 between SynRFP and SynCAV1.**

Figure 5. Neuron-targeted expression of caveolin-1 (Cav-1) enhances expression of Disrupted-In-Schizophrenia-1 (DISC1) and synaptic proteins in human differentiated primary neurons.

The human neurons were differentiated from the Craig Venter 4a (CV4a) neuronal stem cells (NSC), which are derived from human induced pluripotent stem cells (PSCs). Neurons were differentiated in differentiating media for 3-4 weeks. After differentiation, neurons were infected by a letivirus containing the Cav-1 driven by synapsin promoter (HIV-synCAV1) for 72h. SynGFP served as control vector (10^9 viral particle from both vectors). Homogenates were immunoblotted for Cav-1, DISC1, synaptobrevin, PSD95, synaptophysin, syntaxin1, neurexin and GAPDH **(A)**. Western blots quantification showed over-expression of Cav-1 protein **(B)**. Syncav1 also significantly enhanced the protein expression of DISC1 **(C)**, and other synaptic proteins: Synaptobrevin **(D)**, PSD95 **(E)**, Synaptophysin **(F)**, syntaxin **(G)** and neurexin **(H).**

***p<0.05 between WT and SynCAV1.**